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Richard Thibodeau[2] and H. Macy

ROQUEFORT CHEESE originated in France centuries ago. It quickly won the favor of the French people, and its reputation soon spread abroad. It was made originally from sheep's milk, as it is today. Because the supply of sheep's milk is rather limited and because the cheese needs a long period of ripening, extending from ten to twelve months, it has always commanded high prices on the market. Therefore, it has long been considered as a luxury.

It is surprising that this ancient type of cheese, with such a fine reputation, has been so little investigated. Except for a few chemical observations, up to the end of the last century scientists had almost completely ignored Roquefort cheese. The fact that the secret of its manufacturing had been guarded so jealously might have restricted research. However, with the beginning of the twentieth century, Roquefort cheese attracted the interest of the scientist. The microbiologists made the first major contributions by identifying the fungi responsible for the typical ripening of Roquefort cheese. The chemists followed with studies on the chemical changes occurring during the ripening of the cheese.

The cheese industry later proved that a cheese of the same degree of excellence as Roquefort cheese could be manufactured from the less expensive cow's milk. By virtue of the laws and patents protecting the genuine Roquefort cheese made from sheep's milk, this new type of cheese had to be called by a different name. Accordingly, "Bleu" or "Blue" cheese was adopted most commonly to designate Roquefort cheese made from cow's milk. Blue cheese could be sold cheaper than genuine Roquefort cheese since the raw material used to make it was more readily available. However, the long period of ripening still remained a stumbling

[1] Data in this bulletin were taken from a thesis submitted by the senior author to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy granted June, 1940.

[2] École de Laiterie de St.-Hyacinthe, Quebec, Canada.

block, together with the expense of storage, which made it necessary to sell it at prices too high to be considered popular.

Accordingly, attempts have been made to induce Roquefort or Blue cheese to ripen more quickly, without impairing its quality. Ripening at higher temperatures, homogenization of the milk intended for its fabrication, and addition of animal lipase to the curd have been tried successively for this purpose. Each has given, to a certain extent, encouraging results. Since the ripening of Roquefort or Blue cheese is mainly the result of the physiological activities of *P. roqueforti,* it seems that these activities should be investigated first of all and on a scientific basis. After a thorough knowledge of the enzymes of the principal ripening agent has been acquired, it might be possible to use these enzymes in practical studies of ripening with much better chances for success.

The studies reported here have been undertaken with the idea of accomplishing one step in this direction. It is not claimed that this work is complete by any means, because among the many enzymes *P. roqueforti* produces, only protease and lipase have been studied to some extent. It is only hoped that these studies may serve as a starting point for more extensive studies of the enzymes of *P. roqueforti* and their application to the shortening of the ripening period of Roquefort or Blue cheese.

The literature pertaining to the enzymes of *P. roqueforti* is quite limited but there is a tremendous volume of information concerning the enzymes of microorganisms in general. Thibodeau (44) in 1940 reviewed the pertinent literature thoroughly so no attempt will be made to duplicate that contribution in this bulletin.

Experimental Methods

CULTURAL STUDIES

Cultures Used

Four strains of *P. roqueforti* have been used in these studies. They will be designated hereafter as cultures A, B, C, and D. These cultures were obtained from the following sources:

Culture A—Isolated from a genuine French Roquefort cheese.

Culture B—Isolated from a Minnesota Blue cheese.

Culture C—Stock culture collection, Dairy Bacteriology laboratory, University of Minnesota.

Culture D—Furnished by Doctor Charles Thom, Principal Mycologist, United States Department of Agriculture.

The first three strains were identified by the writers as typical of *P. roqueforti.* Transfers from these cultures were sent to Dr. Thom who confirmed the diagnosis.

Other molds used in the experiments were obtained from the dairy bacteriology laboratory, University of Minnesota.

Medium for Stock Cultures

Most of the media used by other investigators were tried, as well as many modifications. After extensive preliminary investigations, the following medium was found to give the most luxuriant growth and toughest mycelial felt:

	grams		grams
Sucrose	30.0	Potassium chloride	0.5
Sodium nitrate	1.75	Ferrous sulphate	0.01
Dipotassium phosphate	1.0	Agar	1.0
Magnesium sulphate	0.4		

Skim milk	50.0 ml.
Water	950.0 ml.

This medium had a reaction of *p*H 7.2. Unless otherwise stated, it was used for the production of mycelium, and will be termed hereafter the "standard medium."

Temperature of Incubation

Since, at times, the material used was too voluminous to be kept in the incubators, all of the experiments, pertaining either to growth or enzyme studies, have been carried out at room temperature. This varied from 20° to 28° centigrade.

Oxidation-Reduction Potentials

The readings were obtained by means of a Model 3D Coleman glass electrode potentiometer. The electrodes were made from soft glass tubing. Into one end of each tube a one-inch piece of 22-gauge platinum wire was sealed. These electrodes were boiled for 15 minutes in concentrated nitric acid and washed thoroughly in distilled water each time before they were used. The cultures were grown in 500 ml. Erlenmeyer flasks containing 200 ml. of culture medium. The flasks were closed with rubber stoppers in which six holes had been bored. An electrode was introduced into each of three holes, hanging just above the culture medium. The other three holes were plugged with cotton. The complete assembly was then sterilized in the autoclave for 20 minutes at 15 pounds pressure.

After sterilization a salt bridge was introduced in each flask in the fourth hole of the stopper. The electrodes were lowered into the medium and filled with mercury. Holes five and six were left plugged with cotton so as to simulate as closely as possible conditions existing in ordinary culture flasks stoppered with cotton. The salt bridges were made from pieces of soft glass tubing bent at one end into the form of a fishhook. At first they were filled merely with agar saturated with potassium chloride and kept in a saturated solution of potassium chloride until ready to use. However, these bridges were not absolutely sterile and brought contamination into certain rich media. In order to eliminate this trouble, the salt bridges had to be sterilized before being introduced into the culture flasks. Therefore, the bent tubes, in necessary numbers, were forced into a cotton plug which was introduced into the mouth of a flask containing the melted potassium chloride-agar. A short piece of rubber tubing was added to the upper end of each tube, and the agar was drawn up until both the glass tube and the rubber tubing were full. A clamp was then used to close the rubber tube, thus preventing the agar from flowing back. The whole assembly was sterilized in the autoclave. When cold, the salt bridges were transferred as aseptically as possible to culture flasks. Culture media were allowed to remain at least 12 hours before inoculation to allow the oxidation-reduction potential of the medium to reach a constant level.

For potential readings the following connections were made. One end of a copper wire was immersed in the mercury filling the tube which held the electrode, and the other end was attached to one of the poles of the potentiometer. The circuit was com-

pleted by connecting the salt bridge to the potassium chloride reservoir through a rubber tubing. The pieces of rubber tubing at the upper end of the salt bridges were kept in place during the whole experiment, in order to prevent desiccation of the agar in the bridges. They were removed only to make the connections when readings were made. During the course of the experiments, great care was taken to avoid shaking the flasks so as to prevent absorption of oxygen into the medium.

STUDIES OF ENZYMES AND THEIR ACTIVITIES

Production of Mycelium for Enzyme Studies

The production of mycelium for enzyme studies was carried out in 2800 ml. Fernbach flasks to which 300 ml. of standard medium were added. The flasked medium was autoclaved at 15 pounds pressure for 20 minutes. After sterilization and cooling, each flask was inoculated with 2 ml. of a heavy aqueous suspension of spores from a young (four to eight days old) culture grown on Czapek's agar.

The cultures were allowed to develop at room temperature until a luxuriant growth with marked sporulation had taken place which took about five days. Then the culture medium was poured out of the flask carefully so that the mold pellicle was left behind in the flask and thus obtained easily. The mycelium from each of the strains being studied was assembled in one flask and washed three times with distilled water. It was then pressed between the hands to eliminate as much water as possible. Following this it was kneaded so as to compact the mycelium as much as possible, after which it was spread over filter paper. It was allowed to dry on the paper for 24 hours. The dry mycelium was then ground in a mortar until a fine powder was obtained. The powder was kept in a desiccator over calcium chloride until ready to use. Usually, 0.05 g. of this powder was added to each 10 ml. of substrate. Preliminary work showed that mycelium prepared by the "acetondauerhefe" (1) method was not any more active than the powder obtained by the method described above.

Substrates for Enzyme Studies

For studies of proteolysis—All proteolysis studies, except in a few instances which will be pointed out later, were carried out on a substrate containing 1 per cent casein. This was prepared by dissolving the necessary amount of Difco isoelectric casein in

a dilute sodium hydroxide solution, and then adjusting the reaction to the desired *p*H. When a buffer was used, a 2 per cent casein solution was prepared. When needed, 5 ml. of this solution were mixed with 5 ml. of buffer solution, thus giving a final casein concentration of 1 per cent. The 2 per cent casein solutions, which were used for optimum *p*H determinations, were prepared for each experiment separately. For studies where a buffer was not needed, a casein solution with final concentration of 1 per cent was prepared in large amounts, bottled, and sterilized, thus providing a substrate of uniform composition for all experiments.

For studies of lipolysis—All lipolysis studies were carried out on a 3 per cent butterfat emulsion. Butter oil was obtained from fresh, unsalted butter made from sweet cream. The necessary amounts of oil, water, and buffer solution were mixed together and enough gum arabic was added to give a final concentration of 0.5 per cent. The mixture was heated to about 160° F. for 10 minutes and then homogenized with a hand homogenizer. Stable emulsions were obtained in this way, although with time some creaming took place. Large amounts were prepared at one time, bottled, sterilized, and stored for further use. For optimum *p*H studies, however, an original emulsion containing 6 per cent oil, 1 per cent gum arabic, and no buffer was prepared separately for each experiment. A final 3 per cent oil concentration was obtained by mixing, when needed, 5 ml. of emulsion and 5 ml. of buffer solution.

Plan of Experiments on Proteolysis and Lipolysis

When optimum *p*H studies were made, either in the proteolysis or lipolysis series, a substrate was prepared as mentioned previously with twice the necessary concentration, but made up to volume only after it had been brought down to the lowest *p*H needed. The proper number of 5 ml. samples was then withdrawn and the samples placed in test tubes or flasks as required. Then the *p*H was brought up to the next level desired, using sodium hydroxide solutions of varying concentrations so that one or two drops would be sufficient (thus causing a minimum dilution of the substrate), the proper amount of substrate withdrawn, and so on until the highest *p*H of the series was attained. The samples withdrawn were placed in 20 ml. test tubes for the proteolysis series and in 125 ml. Erlenmeyer flasks for the lipolysis series. In each tube or flask 5 ml. of the buffer solution were added, thus

giving the final desired concentration of the substrate. In the proteolysis studies, two digests and one blank were prepared for each *p*H of the series, and for the lipolysis studies, two digests and two blanks were prepared.

Digestion was allowed to proceed at room temperature, and the containers agitated from time to time. After digestion, in the proteolysis series all of the samples were boiled for five minutes; in the lipolysis series the enzyme was inactivated by the addition of 50 cc. of a mixture of alcohol and ether.

The blanks in all cases were prepared exactly in the same way as the digests, except that the mycelium was added only at the end of the digestion period.

Buffers

For proteolysis studies—Casein was found to have enough buffering capacity between *p*H 6.0 and 7.0 to render unnecessary the use of a buffer when operating in this range, which was the case for all proteolysis studies outside of optimum *p*H experiments.

For optimum *p*H studies the buffer used was a mixture of potassium dihydrogen phosphate and sodium monohydrogen phosphate solutions in concentrations twice as high as those indicated by Gortner (16). The two solutions were prepared separately and each different *p*H of the series obtained by mixing the necessary quantities of the two solutions until the desired *p*H was obtained, as indicated by the titration electrode.

The *p*H in all cases was checked after all the ingredients (i.e., substrate, buffer if added, and mycelium) had been added and the reading obtained at this stage was taken as final. For example, in an experiment on optimum *p*H studies, a series was set up covering the range of *p*H 3.0 to *p*H 8.6, at intervals of 0.2 of a *p*H unit. A series of 5 ml. samples of substrate (2 per cent casein solution) covering this range was prepared, following the reaction by means of the titration electrode. Then, to each tube were added 5 ml. of the buffer solution of the corresponding *p*H. The mycelium was added, and after the samples had stood a couple of hours, the *p*H was checked by means of a glass electrode potentiometer.

For lipolysis studies—The substrate used had no buffering capacity, therefore a buffer had to be used in all cases.

For the first few experiments a phosphate buffer was used, having the same composition as the one indicated under proteolysis studies. Since this buffer was proven to be rather un-

satisfactory for lipolysis studies, it was abandoned for a sodium acetate-acetic acid buffer, according to directions of Willstätter and Waldschmidt-Leitz (54). This buffer was prepared as follows: a 0.1 N sodium acetate solution was obtained by mixing a 0.1 N acetic acid solution with an equal volume of 0.1 N sodium hydroxide solution. For studies other than optimum *p*H studies, this mixture was brought to *p*H 6.5 by adding the necessary amount of 0.1 N acetic acid solution. The substrate was prepared by mixing the 6 per cent emulsion and this buffer in the proportions of 1:1. For optimum *p*H studies, the procedure was the same as that used in the study of proteolysis. The *p*H also was checked in the same manner.

Preservatives

Bacterial growth was checked by the addition of 1 per cent toluene to proteolytic digests, and 1:1500 formaldehyde (36, 37) to lipolytic digests.

Determination of Proteolytic Activity

The proteolytic activity was determined by titrating the products of digestion of casein by Sörensen's formol titration (40), using Brown's technique (7) (recommended by the Society of American Bacteriologists). The method was modified in that the end points of titration were determined by means of the glass electrode, instead of phenol red indicator. At first, titrations were made on one milliliter amounts of substrate, as recommended by Brown, but later it was found that the results were much more uniform if titrations were made on 5 ml. amounts. Working with a glass electrode, a minimum volume of 50 ml. was needed (45 ml. of distilled water were added to 5 ml. of substrate), thus necessitating a change in the proportions of formalin indicated by Brown. Preliminary work showed that maximum titration was obtained when the different ingredients were used as follows:

5 ml. substrate, 45 ml. distilled water, 10 ml. commercial formalin.

The initial and end points of titration were determined, according to Brown, by plotting the titration curves of casein digests. The initial point of titration (i.e., the reaction to which the digest must be brought before addition of formalin) must be within the isoelectric zone of the amino acids present in the digest. As shown by figure 2, *p*H 7 was found to be within this zone and accordingly was selected as the initial point of titration.

The end point of titration must be within the zone of neutralization of the methylene derivatives of the amino acids, and the zone is recognized on the resultant curve (curve obtained by subtracting the formaldehyde titration curve from the curve obtained by the formol titration of the substrate) when it reaches a position parallel to the *p*H axis. Figure 2 shows that *p*H 8.5, the selected end point of titration, lies within this zone.

Brown (7) in his method did not make provisions for the use of a blank. However, raw casein contains some free, titratable groups (17, 21). Therefore, the values obtained by this titration must be subtracted from those obtained on the digests. Accordingly, the proteolytic activity of mycelium was expressed as the difference between the average total formol titration (i.e., formaldehyde plus substrate) of the digests and the total formol titration of the blank.

When a buffer was added to the substrate, it was precipitated, before titration was performed, by adding barium chloride to the substrate which was previously made alkaline.

The method outlined above was compared with the Van Slyke method (48) and was found to give results somewhat higher than the latter. It was also found that the duplicates checked more closely by the first method. Duplicates usually checked within 0.00 to 0.05 ml. of 0.1 N sodium hydroxide when 5 ml. of the digests were titrated.

Determination of Lipolytic Activity

Lipolytic activity was determined by titrating the fatty acids liberated, with 0.1 N alcoholic potassium hydroxide in the presence of alcohol and ether (38).

As mentioned previously, the substrate, in 10 ml. amounts, was placed in 125 ml. Erlenmeyer flasks. At the end of the digestion period, 50 ml. of a mixture of alcohol and ether (1:1) were added to each flask, and the free fatty acids titrated with 0.1 N alcoholic potassium hydroxide, using phenolphthalein as the indicator (10 drops). Lipolytic activity was expressed as the difference between the average titration of the digests and that of the blanks.

Expression of Enzymatic Activity

Enzymatic activity, in all cases, was expressed in milliliters of alkali, that is, the number of milliliters of 0.1 N alkali necessary

to neutralize the products due to the disintegration produced in 10 ml. of substrate by 0.05 gm. of mycelium.

Quick Test for Detection of Enzymes

Hédon (20) developed a quick, qualitative test to detect the presence of trypsin. This test was used, after modification, to detect in cultures of *P. roqueforti,* the stage of growth at which lipase and protease appeared in the culture medium. The modified method was as follows. To 600 ml. of substrate were added 2 ml. of bromcresol purple (0.5 per cent aqueous solution of the sodium salt) and the reaction brought to *p*H 6.0 in the casein solution and to *p*H 6.5 in the fat emulsion. Each day, portions of culture medium were withdrawn from growing cultures. Then eight test tubes were prepared, four containing 10 ml. of casein solution each and four containing 10 ml. of fat emulsion each. To two test tubes of each set above, one ml. of culture medium was added, and to the remaining tubes, one ml. of the boiled culture medium was added, the latter serving as blanks. The tubes, set up in a rack, were placed in the incubator at 37° C. and examined from time to time for color changes.

The substrates used for these experiments were not buffered, in order to allow minor changes in acidity to be easily detected.

Cheesemaking Experiments

Three batches of Blue cheese were made to determine the effect, on the rate of ripening, of adding powdered mycelium to the curd. The procedure followed for making the cheese was that used by the Dairy Division of the University of Minnesota. Mold powder, prepared according to the Hussong and Hammer method (22), was added to all of the cheeses, whether mycelium was added or not.

Cheese Ripening Studies

The rate of ripening of the cheeses was followed by determining periodically the extent of protein and fat decomposition and also by making observations on body and texture, flavor development, and extent of mold growth. The extent of protein decomposition was determined by the Van Slyke amino nitrogen determination (48) method. The extent of fat hydrolysis was determined by the steam distillation of duplicate samples of cheese, titrating only the soluble volatile fatty acids.

Presentation of Data

CULTURAL STUDIES

Culture Media for the Growth of *P. roqueforti*

Studies were carried out on certain factors which might influence the growth of *P. roqueforti.* The purpose of these studies was to obtain a culture medium which would stimulate the growth of *P. roqueforti* so that an abundant development of mycelium might be obtained in the form of a thick, resistant felt which could be easily and completely recovered.

The preliminary studies clearly revealed that while some of the common molds may grow luxuriantly on Czapek's solution, the medium had to be modified so as to meet certain requirements if an abundant growth was to be obtained with *P. roqueforti.* The same studies indicated also that different strains of *P. roqueforti,* on a given medium, did not grow to the same extent. In the present instance, strain "A" was more difficult to grow satisfactorily than the other strains. Accordingly, more detailed observations had to be made on some of the factors most likely to affect growth.

The character of the growth in the experiments to be reported was recorded according to the appearance of the cultures, the yield of mycelium, the formation of a pellicle, and the resistance of the pellicle.

The following code will be used to express the character of the growth:

0 = none
1 = scanty
2 = poor
3 = moderate
4 = good
5 = abundant
6 = very abundant

* = pellicle formed
† = pellicle formed, but sank into the medium
(No sign after the figures means that growth was diffuse)

The medium used as a basis for further studies was Czapek's solution with the following composition:

	grams		grams
Sucrose	30.00	Magnesium sulphate	0.50
Sodium nitrate	2.00	Potassium chloride	0.50
Dipotassium phosphate	1.00	Ferrous sulphate	0.01
Water	1000.00 ml.		

P. roqueforti grew poorly on this medium. No definite pellicle was formed while most of the growth appeared throughout the liquid. Complete recovery of the mycelium could be effected only by the use of a filter.

The first factor to be studied in relation to the improvement of growth was the influence of the reaction of the medium. Lots of Czapek's solution were brought to different reactions, sterilized, and inoculated, after cooling, with *P. roqueforti.* After growth had been allowed to proceed for 10 days it was found that the reaction had no influence on growth within the ranges used, as shown below. The same results were obtained when the experiment was repeated a number of times, other media being used also.

pH	Growth
4.0	2
6.0	2
7.5	2

Therefore, it became apparent that some factor other than the reaction should be investigated. Observations were then made on the influence of the source of carbon. Experiments were carried out where different sugars, fats, or fatty acids (adjusting the reaction with sodium hydroxide) were used in Czapek's solution in amounts providing about the same amount of carbon as when sucrose was used. The results outlined below indicated that sucrose as a source of carbon was as satisfactory as any used and better than some. Growth was poor and no pellicle was formed.

Source of Carbon	Growth	Source of Carbon	Growth
None	0	Lactose	2
Sucrose	2	Tributyrin	2
Glucose	2	Ethyl acetate	1

The next step was an investigation of the influence of the source of nitrogen. Various nitrogenous compounds, organic and inorganic, were used in Czapek's solution, in place of sodium nitrate. These experiments were repeated several times, using the nitrogenous compounds in such quantities that the amount of nitrogen furnished to the culture medium would be the same as when 2 g. of sodium nitrate were used. The results thus obtained are outlined below. They showed that the source of nitrogen had a marked influence on the growth of *P. roqueforti.* They also showed that as far as *P. roqueforti* was concerned, sodium nitrate was one of the poorest sources of nitrogen that could be used. They further indicated that, in general, an organic source

of nitrogen was more effective for promoting growth but was not indispensable, since the highest yield of mycelium was obtained when ammonium sulphate was the source of nitrogen. When skim milk was used, the organism formed a felt, but only around the walls of the containers. Although there was a tendency toward the formation of a pellicle in the media containing an organic source of nitrogen, the pellicle was weak and soon sank into the medium, so that it was as difficult to recover the mycelium from such cultures as it was when a diffuse type of growth occurred. It should also be emphasized that when the mycelium had to be recovered by filtration, the precipitates occurring in the culture media were recovered as well, thereby causing errors in the estimation of the enzymatic activity of the mycelium.

Source of Nitrogen	Growth	Source of Nitrogen	Growth
None	0	Peptonized milk	3†
Sodium nitrite	1	Skim milk	3*
Sodium nitrate	2	Ammonium chloride	4
Ammonium oxalate	3	Urea	4†
Ammonium lactate	3	Ammonium sulphate	5
Peptone	3†		

Traces of yeast extract when added to plain Czapek's solution stimulated growth remarkably, but provoked a diffuse growth throughout the medium, instead of the formation of a felt at the surface, as desired.

It was thought that the organism might need some support for its development or perhaps more surface. Accordingly, sterile pieces of glass, slices of cork, and drops of paraffin were added to Czapek's solution after inoculation. Where glass was used, the fragments of glass were large enough to extend above the surface of the medium. The mold grew heavily on the slices of cork and to a lesser extent around the drops of paraffin and the pieces of glass. It had been observed previously that growth was often more extensive along the walls of the flasks. These materials, however, promoted only a discontinuous growth, i.e., on or around the pieces of solid material, and furthermore, the mycelium was not easily recovered, especially from the cork and paraffin. Although expectations were not quite fulfilled, the results obtained threw some light on the requirements of *P. roqueforti*. Growth was stimulated to different degrees by the solid materials, the order of efficiency being cork>paraffin>glass. It was noted that the growth-promoting capacities of these materials were decreasing as the materials used were more amorphous. Accordingly, it was thought that if a colloidal material were added,

it would provide still more surface and at the same time be an integral part of the nutrient solution. At the same time it would not interfere with the recovery of the mycelium. Accordingly, agar in 0.1 per cent concentration was added to Czapek's solution. The results were surprisingly good. A uniform pellicle consistently developed at the surface of the media containing agar.

Medium	Growth
Czapek's solution	2
Czapek's + 0.1 per cent agar	4*

Growth was typical of *P. roqueforti* when compared with cultures growing on solid Czapek's medium. Although the pellicles were not very heavy, they were resistant enough to be handled without losses and could be recovered simply by pouring out the medium, the use of a filter being no longer needed. Without doubt, an important step had been accomplished. There remained the need to increase the yield of mycelium. For this purpose, different sources of nitrogen, organic and inorganic, were added to the new medium, that is, Czapek's solution containing 0.1 per cent agar. The results, summarized below, showed that the heaviest growth was obtained when milk and agar were added to Czapek's solution (in media 4, 5, and 6 the sodium nitrate was completely replaced by the nitrogenous compounds indicated). It was also noted that when the culture medium was too rich in organic nitrogen, the mycelium had a tendency to sink into the culture medium, thus decreasing considerably the yield of mycelium.

Medium	Growth
1 — Czapek's	2
2 — Czapek's + 0.1 per cent agar	4*
3 — Czapek's + 0.1 per cent agar + 5 per cent skim milk	6*
4 — Czapek's + 0.1 per cent agar + ammonium chloride	4*
5 — Czapek's + 0.1 per cent agar + ammonium sulphate	4-5*
6 — Czapek's + 0.1 per cent agar + peptone	5†

The medium finally found to answer most adequately the requirements for abundant growth and the formation of a thick, resistant felt had the following composition:

	grams		grams
Sucrose	30.00	Potassium chloride	0.50
Sodium nitrate	1.75	Ferrous sulphate	0.01
Dipotassium phosphate	1.00	Agar	1.00
Magnesium sulphate	0.40		
Skim milk	50 ml.		
Water	950 ml.		

This culture medium has been termed the "standard medium."

The studies reported above indicated that the reaction of the medium and the source of carbon were without noticeable influence on the growth of *P. roqueforti.* Studies on the influence of the source of nitrogen showed that although different nitrogenous compounds improved growth markedly, none was found to favor the type of growth desired. Sodium nitrate, ordinarily used in Czapek's solution, was one of the poorest sources of nitrogen. The desired type of growth was secured only when agar was present in the medium.

Oxidation-Reduction Potential Studies

The cultural studies showed beyond doubt that the growth of *P. roqueforti* was governed not only by the type of nutrients available but also by some secondary factors previously unstudied. These secondary requirements were satisfied when agar was present in the medium. The question naturally arose, therefore, as to what influence the agar, a supposedly inert material, might have on the growth of *P. roqueforti.* It was thought at first that the agar, as a colloidal substance, provided an enormous number of particles on the surface of which growth was catalyzed. From a purely mechanical standpoint, it was considered possible that in a liquid medium containing agar, on account of the viscosity, the sinking of the mycelium into the medium might be prevented, thus allowing the formation of a pellicle and ultimately favoring an increase in the yield of mycelium. However, after closer examination the explanation did not appear to be quite so simple. It was observed that a concentration of 0.05 per cent agar had almost the same beneficial effect as 0.1 per cent and that increasing the concentration of agar up to 1 per cent gave no better results than a concentration of 0.1 per cent. It seemed rather unlikely that such a small increase in viscosity as the one obtained with a 0.05 per cent concentration could alone be responsible for such a tremendous difference in the appearance and extent of the growth. There was still to be considered the possibility that agar might not be an electromotively inert substance and that the beneficial influence might be associated with the oxidation-reduction potential of the medium. Accordingly, redox measurements were made on four representative media:

1. Czapek's solution
2. Czapek's solution plus 0.1 per cent agar
3. The "standard" medium
4. The standard medium, replacing the milk by 0.1 per cent of peptone

The reaction of each medium was adjusted to *p*H 6.0 before sterilization.

This experiment was repeated three times using *P. roqueforti* strains A, B, and D, respectively. Essentially the same results were obtained in all three experiments. Accordingly, complete results are reported only for Experiment 2, which is typical. These results are presented in figure 1 where E*h* is plotted against time. The curves are derived from the readings obtained from one electrode only, since the three electrodes in each medium gave readings which agreed very well.

The *p*H of the medium at various intervals was as follows:

Medium	pH after			
	5 hours	24 hours	36 hours	98 hours
1	7.45	7.38	7.30	7.15
2	7.58	7.51	7.45	7.15
3	7.30	7.10	7.05	6.40
4	7.60	7.42	6.95	.....

As shown by figure 1, the four media gave characteristic curves. The original potential of the media, as shown in the first part of each curve, is considered to be most important. In fact, the data show that the four media classify themselves in order of decreasing original potentials as 1, 2, 4, 3, which is exactly the order into which they arranged themselves from the standpoint of increasing yield of mycelium. The course of each time-potential curve is determined by the extent of the growth, which in turn is apparently regulated by the initial oxidation-reduction potential of the medium. In table 1 are given the initial potential readings (mean of the readings obtained from the three electrodes on each medium five hours after inoculation) of each medium for the three experiments. The regularity of the results should be noted. Medium 1 had an initial oxidation-reduction potential consistently above 400 millivolts. The lag phase on this medium was long and growth was poor and diffuse. On the other hand, when agar

Table 1. Initial Oxidation-Reduction Potentials of the Culture Media

Medium	Eh five hours after inoculation		
	Experiment		
	1	2	3
	Millivolts	Millivolts	Millivolts
1	442	433	446
2	390	384	398
3	245	234	276
4	302	294	328
Mold used	A	B	C

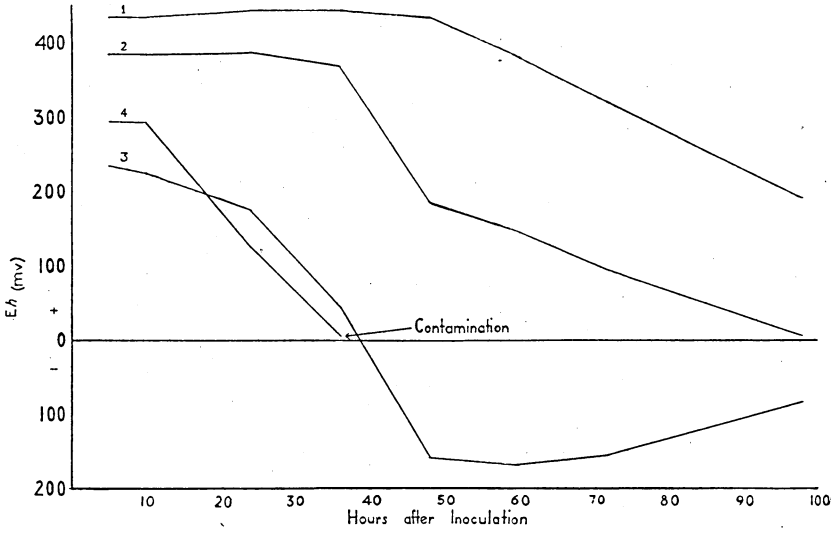

FIG. 1. INFLUENCE OF GROWTH OF *P. roqueforti* ON THE OXIDATION-REDUCTION POTENTIALS OF CULTURE MEDIA

was added to Medium 1 (giving Medium 2) the initial oxidation-reduction potential was lowered below 400 millivolts. The lag phase was shortened and a pellicle, although thin, was formed. Media 3 and 4 had still lower initial potentials. Their lag phase was about the same length, but shorter than in Medium 1 or 2. Growth in Medium 4 developed more rapidly than in Medium 3—which probably accounts for the earlier drop in the oxidation-reduction potential of the medium although the final yield of mycelium was superior in Medium 3.

These results definitely indicated that *P. roqueforti* does not grow readily in a highly oxidized medium, that is, a medium having an initial oxidation-reduction potential above 400 millivolts. When agar was added to the medium, the potential was brought below 400 millivolts and the organism formed a pellicle and grew much more extensively. If, in addition to agar, milk or peptone was introduced into the culture medium, the initial potential of the medium was lowered still more, probably due to these substances containing reducing agents such as cysteine, and the mycelium yield increased in proportion to the decrease in the initial potential. Whether the improvement in the growth of *P. roqueforti* was due solely to a decrease in the initial oxidation-reduction potentials of the media cannot yet be ascertained. It is evident, however, that the results reported in figure 1 and in

table 1 are significant. Further studies should be made using inorganic colloids and inorganic oxidizing and reducing agents.

After this preliminary work was completed, the articles of Jacobi and Schwarz (24) on the beneficial influence of different colloids on mold growth, and of Allyn and Baldwin (2, 3) on the influence of the initial oxidation-reduction potential of the medium on the growth of Rhizobia came to the attention of the writers. Jacobi and Schwarz (24) found that the effectiveness of the colloids studied by them was expressed by the series gold sol>agar>albumin>water glass>methyl cellulose>sulphur> turkish oil. The first sol, which was also the most effective, eliminated the source of nitrogen as being the beneficial agent. On the other hand, the same sol was very probably not a reducing agent, thus emphasizing the importance of the surface factor. Allyn and Baldwin (2) explained the effectiveness of solid particles in liquid media on the ground that "this was due to the trapping of small volumes of the medium where the organisms were able to make the necessary adjustments in the oxidation-reduction potentials of the media." Allyn and Baldwin (3) also found that agar in concentrations of 1:3,500 not only appeared to afford physical advantages to Rhizobia in initiating the growth, due to its colloidal nature, but also to act as a mild reducing agent. Pellicle formation by the same organisms in liquid media was definitely favored by low oxidation-reduction potentials of the media. They also found that such factors as a more available source of nitrogen or a more suitable ion-balance made it easier for the organisms to make the necessary adjustments in the potentials of media too highly oxidized to favor growth. No references concerning the influence of oxidation-reduction potentials on the growth of molds are available in the literature. Accordingly, conclusions must be drawn from rather meager data.

It seems apparent from studies made on the oxidation-reduction potentials of different modifications of Czapek's solution that *P. roqueforti* does not grow readily on a medium having a high oxidation-reduction potential. Agar in concentration of 0.1 per cent decreased the initial oxidation-reduction potential of Czapek's solution below 400 millivolts, materially improved growth, and very definitely caused the formation of a pellicle by the organism. When milk or peptone in addition to agar was introduced into the culture medium, a further decrease of the initial oxidation-reduction potentials of the media resulted and the yield of mycelium was increased. The yield of mycelium on the dif-

ferent media was inversely proportional to the initial oxidation-reduction potentials of the media.

It is possible that when agar and an organic source of nitrogen are added to Czapek's solution, the improvement in growth of *P. roqueforti* may be due to a combination of factors such as lower initial oxidation-reduction potentials, increase in viscosity of the medium or provision of surface where growth is catalyzed, and an immediate, more available source of nitrogen.

STUDIES OF PROTEOLYSIS

Determination of Proteolytic Activity

As mentioned earlier, the disintegration products resulting from the activity of the proteolytic enzymes on casein were titrated by Brown's (7) modification of Sörensen's (40) formol titration. Brown's technique was further modified in order to adapt it to the titration electrode which was used instead of an indicator to determine the end points of titration.

The first operation performed was the determination of the initial point of titration, which should be located in the isoelectric zone of the amino acids present in the digest. For this purpose, 1 per cent casein solutions at *p*H 7.0 were allowed to digest for different lengths of time with about 0.5 per cent of mycelium powder added to the solution. After digestion the solutions were boiled, filtered, and the titration curves determined on 1 ml. samples, using a N/20 sodium hydroxide solution. Until the optimum amount of formalin to be added had been ascertained, 8 ml. amounts of formalin were used when necessary.

In figure 2 the results of a typical experiment are reported. In this case, digestion had been allowed to proceed for 10 hours. All the curves in this figure represent the average titration values of three samples. The curve under "first titration" was for the purpose of determining the isoelectric zone of the compounds present in the digestion mixture, and then to select in this zone the reaction to which the sample should be brought before the addition of formalin. The selected reaction is designated as the initial point of titration. Although the reaction represented by *p*H 7.0 was selected, the data showed that any point between *p*H 6.9 and *p*H 7.2 might have been selected.

The next step was the determination of the end point of titration. This point should be situated on the resultant curve (curve expressing the difference between the formol titration of the sample and the titration of formaldehyde) in the region of neutraliza-

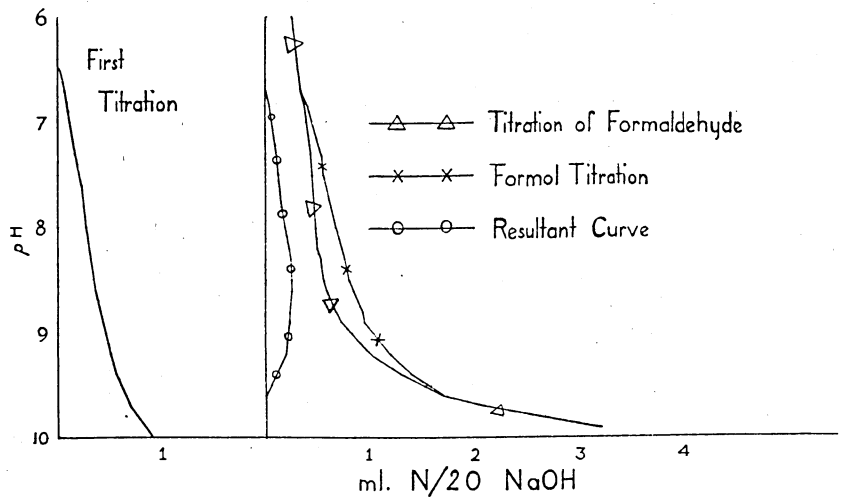

FIG. 2. FORMOL TITRATION OF A CASEIN DIGEST

tion of the methylene derivatives of the amino acids (or digestion products), which region is recognized when this curve reaches a position parallel to the *p*H axis. This region of the resultant curve, which should also represent the maximum titration, varied somewhat in location from experiment to experiment but the selected end point, *p*H 8.5, was always found in this region. The results reported in figure 2 show that in this case the region of neutralization extended from *p*H 8.3 to *p*H. 8.9. It was observed that the further the digestion was advanced, the narrower this region was.

Undigested casein contains some formol-titratable groups (17, 21, 30). Accordingly, when proteolytic activity is determined, the formol titration of a blank must be subtracted from the formol titration of the digest. An idea of the magnitude of the formol titration of raw casein may be gathered from the curve in figure 3. The results reported in this figure are those obtained on a 1 per cent raw casein solution. In agreement with the above, proteolytic activity was expressed by the difference between the total titration of the digests and that of the blanks. Thus the causes of error due to the reactive groups, contributed not only by the casein but also by the mycelium, were eliminated since it was proved experimentally that mycelium also contains some groups reacting with formaldehyde.

It should be mentioned here that the experiments on the determination of the initial and end points of titration were carried

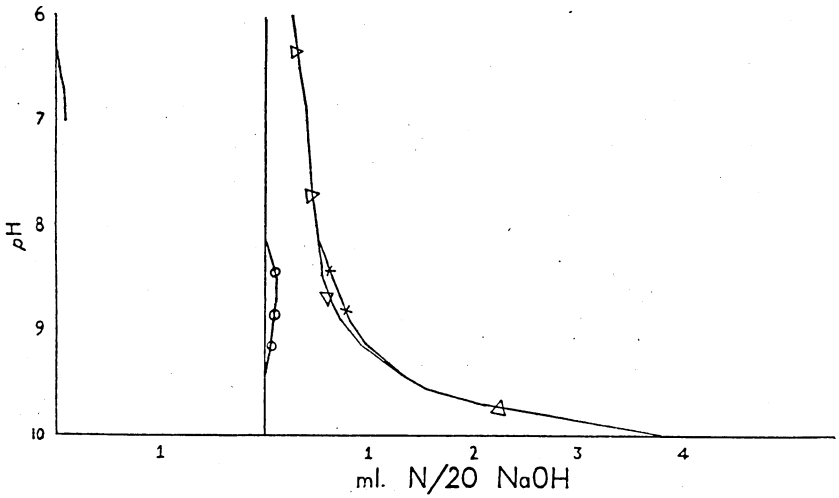

FIG. 3. FORMOL TITRATION OF A BLANK

out on unbuffered substrates. Accordingly, for a large part of them, such as those reported in figures 2 and 3, the buffers were not precipitated before titration. However, this treatment for precipitation of the buffers was applied in a few instances. When this was the case, it was observed that a light precipitate occurred. This precipitate was filtered out. It was also observed that the titration results on these treated samples were absolutely the same, compared with untreated samples of the same source.

The third step in the establishment of the new technique was the determination of the optimum amount of formalin to be added, that is, the amount of formalin which would give the maximum titration. Table 2 shows that the maximum titration was obtained when 10 ml. of formalin were used and the end point of

Table 2. Optimum Concentration of Formalin

Formalin	End point of titration pH 8.0		pH 8.5		pH 9.0	
ml.	Proteolytic activity calculated by difference between formol titration of digests and titration of					
	Blank	HCHO	Blank	HCHO	Blank	HCHO
	ml. N/20 NaOH					
8	.19	.23	.17	.27	.19	.24
10	.25	.29	.31	.36	.30	.28
12	.15	.26	.22	.31	.10	.31
13	.19	.27	.20	.30	.10	.35
14	.17	.27	.18	.30	.12	.35
15	.29	.29	.29	.35	.....	.....

titration was *p*H 8.5. Table 2 shows that this was true whether proteolytic activity was calculated as the difference between the formol titration of the digests and the titration of formaldehyde or the difference between the formol titration of the digests and the formol titration of the blanks. The results reported in table 2 are the average titration values of three casein solutions allowed to digest for 24 hours. The method, based upon these results, can be outlined as follows:

1. 1 ml. substrate (2 ml. when the buffers were precipitated, because the original solution was diluted to twice its original volume during the operation).
2. 50 ml. of distilled water.
3. Reaction brought to *p*H 7.0.
4. 10 ml. of commercial formalin added.
5. Titrated to *p*H 8.5 with a N/20 solution of sodium hydroxide.
6. Proteolytic activity expressed by the difference between the formol titration of the digests and the formol titration of the blanks.

After this method had been in use for some time, it was considered desirable to increase the amounts of substrate used for titration, in order to minimize the experimental errors. Accordingly, it was planned to use 5 ml. amounts of substrate and to titrate with N/10 sodium hydroxide. Since the amount of substrate was changed it was thought necessary to check over the amount of formalin to be used under the new conditions. For this purpose a casein solution at *p*H 6.0 was allowed to be digested for 24 hours with 0.3 per cent of mycelium powder. After the digestion period the solution was boiled and filtered, after which 5 ml. amounts were titrated with different amounts of formalin. The results reported in table 3 are the average titration values for three samples, the proteolytic activity being calculated as the dif-

Table 3. Optimum Concentration of Formalin

Formalin	End point of titration pH 8.0	pH 8.5
ml.	ml. N/10 NaOH	
1	.45	.52
8	.79	.89
10	.83	.93
12	.84	.93
14	.83	.91
18	.86	.92
10*	.83	.91

* This sample was treated for precipitation of buffers.

ference between the formol titration of the digests and the formol titration of the blanks. Here again, the maximum titration was reached when 10 ml. of formalin were added and when the end point of titration was at *p*H 8.5. Accordingly, after the last modification the method differed from the outline given on the preceding page in the following points:

1. 5 ml. substrate (10 ml. when the buffers were precipitated).
2. 45 ml. distilled water (40 ml. when an original 10 ml. sample was used).
3. Titrated with a N/10 solution of sodium hydroxide.

A comparison was made between the formol titration method (last modification) and the Van Slyke manometric method. For this purpose a 1 per cent cheese dispersion was used. Table 4 gives the average results of duplicate determinations. This table shows that there was good agreement between the two methods, although the results were somewhat higher with the formol titration method. It should be mentioned that since there was no blank available, the amino nitrogen in the formol titration was calculated, as in Brown's original method, from the difference between the formol titration of the cheese dispersion and the titration of the formaldehyde, and that all the products thus titrated were calculated as mono amino acids.

Table 4. Comparison Between the Modified Formol Titration Method and the Van Slyke Manometric Method for the Determination of the Amino Acids

Method	Amino nitrogen per gram of cheese
	Milligrams
Formol	7.66
Van Slyke	7.42

When the end points of titration were determined by means of the glass electrode, the formol titration gave consistent results. The results obtained by this method checked closely with those obtained by the Van Slyke manometric method.

Optimum Reaction for Protease Activity

Several experiments were devoted to the determination of the reaction favoring optimum activity of the protease of *P. roqueforti*. The results from these experiments agreed quite well as to the point or region in the *p*H scale at which protease activity was most favored. However, since these experiments were all individual trials, that is, made on substrate solutions prepared for each experiment and using mycelium from different strains or produced at different times, it is felt that a single curve should not

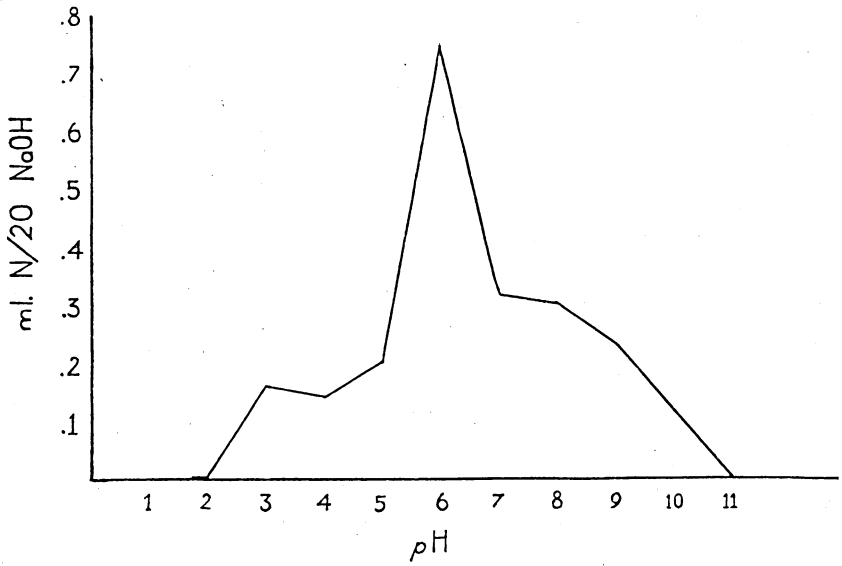

FIG. 4. OPTIMUM ACTIVITY OF PROTEASE

be derived from all those curves by statistical computations, but rather that a few curves should be reproduced individually.

In figure 4 the proteolytic activity of *P. roqueforti,* strain "A," after a 60-hour digestion period is plotted against the reaction. The *p*H range covered was from *p*H 1.75 to *p*H 11.0, at intervals of one *p*H unit. The curve shows the peak of activity to be definitely at *p*H 6.0. Figure 5 shows the results from another experiment where a shorter *p*H range was covered, but at intervals of 0.2 of a *p*H unit, using the mycelium from strain "B" and a digestion period of 40 hours. The curve shows that one should not speak of an optimum point but rather of an optimum range for the activity of protease. In this case the optimum range was between *p*H 5.7 and *p*H 6.2, or even between *p*H 5.3 and *p*H 6.6. The same figure also shows that the phosphate buffer had a somewhat retarding influence on the activity of the protease but had no significant influence on the location in the *p*H scale of the optimum range.

It was thought that by using a shorter period of digestion, a more definite optimum point might be obtained. Accordingly, three series of substrate samples were prepared and allowed to digest for 16, 40, and 70 hours, respectively. The findings (Fig. 6) were the opposite of those expected, the optimum range becoming narrower as the length of the digestion period increased.

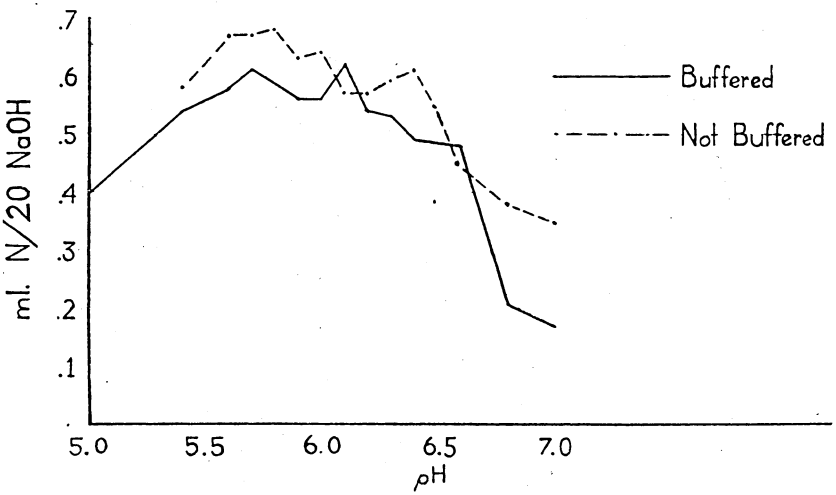

FIG. 5. OPTIMUM ACTIVITY OF PROTEASE

For the experiment reported in figure 7, the last modification of the formol titration method was used, that is, titration was performed on 5 ml. amounts of substrate with a N/10 solution of sodium hydroxide. The curves, which are much more regular, prove that the modification was preferable. Curve 1 represents the protease activity after a 36-hour digestion period. The results confirm those reported in figures 4, 5, and 6 as to the optimum range. Curve 2 also confirms the results reported in figure 5, namely, that the phosphate buffer retarded somewhat the activity of the protease without, however, displacing the range of optimum activity.

During the course of this work it was suspected that toluene, the preservative used, was rather ineffective in checking the bacterial activity in the substrate when long periods of digestion were used. This is the reason why the mycelium was not added to the blanks at the same time it was added in the digests. If the mycelium had been added at the beginning of the digestion period, the blanks would have had to be boiled in order to inactivate the enzyme. At the same time, however, most of the bacteria would have been destroyed, thus providing conditions dissimilar to those existing in the digests. Accordingly, the blanks, like the digests, were boiled only at the end of the digestion period (when the mycelium was added to the blanks), thus allowing for the same conditions from the bacterial standpoint in both digests and blanks. Thus if bacteria were the cause of some protein decomposition, the portion of the proteolytic activity due to their action

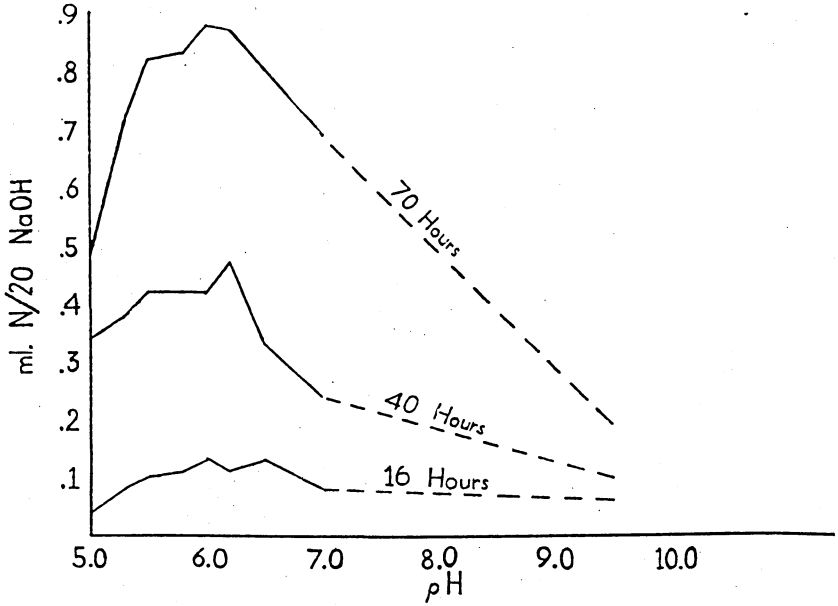

FIG. 6. INFLUENCE OF LENGTH OF DIGESTION PERIOD ON OPTIMUM REACTION

was eliminated when the titration of the blanks was subtracted from the titration of the digests. When the digestion period was less than 20 hours, no protein decomposition was found to be brought about by bacteria. However, as the length of the digestion period increased, the amount of protein breakdown due to bacterial activity increased. The extent of protein decomposition brought about by bacteria was calculated as the difference between the total titration of the blank (i.e., formol titration) and the titration of the formaldehyde. An idea of the bacterial activity on the substrate during a 36-hour period of digestion may be gathered from Curve 3 in figure 7. The fact that the optimum activity of the bacteria was taking place at or around *p*H 7.0 confirms the results of other investigators on the optimum reaction for the activity of the protease of bacteria (12, 13, 25, 53, 55). It also proves that bacteria did not interfere with the optimum proteolytic activity of the mycelium, since the optima were different and since the activity due to bacterial action was subtracted when the titration of the blanks was subtracted from the titration of the digests.

From the data reported here it may be stated that the protease of *P. roqueforti* did not attain its optimum activity at a definite

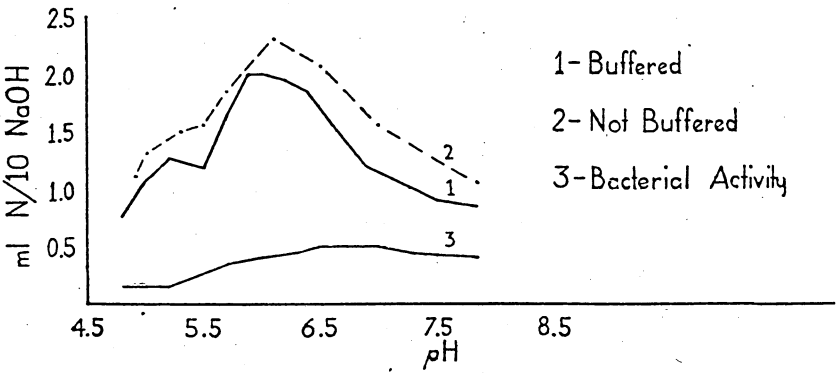

FIG. 7. REACTION FAVORING OPTIMUM ACTIVITY OF THE PROTEASE OF *P. roqueforti* AND OF BACTERIA

point in the *p*H scale but rather that the optimum activity was favored over a range lying roughly between *p*H 5.8 and *p*H 6.3 (figure 7) or even between *p*H 5.3 and *p*H 7.0. These findings are in agreement with those of Waksman (50) and Oshima and Church (35) on other molds. They are, however, at variance with those of Harada (19) and Naylor et al. (34), who found the optimum activity of mold protease to be, respectively, at *p*H 5.2 *(A. oryzae)* and *p*H 5.3 *(P. roqueforti)*.

From an industrial standpoint, the reaction favoring the optimum activity of the protease of *P. roqueforti* is significant. According to the data published by Coulter et al. (9), the reaction of the cheese during the largest portion of the ripening period would be within the range reported here as favoring the optimum activity of the protease, while, save for a few days at the beginning of the ripening period, it would not favor an optimum activity around *p*H 5.3.

The protease of *P. roqueforti,* on a casein substrate and when a phosphate buffer is employed, has its optimum activity at a reaction represented by *p*H 6.0 or, more accurately, in a range lying between *p*H 5.8 and *p*H 6.3.

The reaction corresponding to the optimum activity of the protease is significant in that it means that the reaction of Blue cheese during the longest portion of the ripening period favors the optimum activity of the protease.

The optimum activity of the bacterial protease, under the same conditions as above, was found to be at or around *p*H 7.0, which is in agreement with the findings of previous investigators in the field.

Toluene was shown to be a poor antiseptic when a long period of digestion is used. However, the use of a blank is thought to eliminate the disturbances due to bacterial action, when the protease activity of a given material is investigated. A period of digestion longer than 20 hours is not recommended.

Nature of the Protease of *P. roqueforti*

The protease of molds has been successively identified with all the known animal proteases. Sometimes it has been classified in a separate group between the animal and plant proteases. Recently, its ereptic nature has been particularly emphasized, although some investigators still hold it to be of the trypsin type. At all events, the true nature cannot be ascertained as yet and the debate will continue for some time to come.

As a contribution to the knowledge of the nature of the protease of molds in general and particularly in *P. roqueforti,* a few experiments have been made on this subject.

The first experiment was concerned with the possibility of the peptic nature of the protease, although its optimum reaction indicates it is very improbable. If the protease is tryptic or ereptic in nature, it should be more active on a peptic digest than on a raw substrate; if it is peptic in nature, it should be less active on the peptic digest because the products of the first digestion should cause some inhibition. Accordingly, a large volume of a 2 per cent casein solution was prepared, placed in two bottles, and sterilized in the autoclave. The contents of one bottle were then brought to *p*H 1.8, and 0.1 per cent of an old (exact age unknown) Bacto pepsin powder was added. A portion of the mixture was withdrawn and boiled immediately to serve as a blank. After digestion had proceeded for 17 hours, the digest and blank were boiled again and the formol titration made on triplicate samples of each. The blank was then discarded. The remainder of the peptic digest was brought to *p*H 6.0, as was the other bottle of casein solution which was still sterile. Five 10 ml. samples were withdrawn from each solution (peptic digest and raw casein solution) and placed in test tubes. To three tubes of each series, 0.05 gram of mycelium was added, the other tubes being treated as blanks. Digestion was allowed to proceed for 20 hours, after which the samples were formol titrated in the usual manner, using the last modification of the method, as was done in all experiments to be reported hereafter.

The results reported in table 5 are the average values from

Table 5. Comparative Activity of the Protease of Mycelium on Raw Casein and on Pepsin Digested Casein

Samples	Activity
	ml. N/10 NaOH
Peptic digest	.38
Raw casein plus mycelium	.57
Peptic digest plus mycelium	1.14

three titrations. They show that the protease of the mycelium was twice as active when acting on a substrate which had already been subjected to the action of pepsin. Accordingly, the protease of mycelium might be of the trypsin or erepsin type but it is not of the pepsin type.

Dernby (10, 11) characterized the nature of the proteases present in yeast according to the reaction at which peaks were appearing on the reaction-activity curve. A similar experiment was instituted here with the purpose of finding different optima on different substrates. The procedure was similar to that used in the previous experiment. A 1 per cent buffered casein solution was prepared, placed in two bottles, and sterilized in the autoclave. One bottle was brought to *p*H 7.8 and allowed to digest 20 hours with the addition of 0.1 per cent of trypsin powder (nine years old) after which it was boiled in order to inactivate the enzyme. Then triplicate series of 10 ml. samples were prepared from the trypsin digest and from the second bottle of sterile casein solution. These series covered the range of *p*H 1.8 to *p*H 9.0 at intervals of 0.2 of a *p*H unit. Two of the samples were treated as digests, i.e., they received 0.05 gram of mycelium, and the third sample in each set served as a blank. After digestion had been allowed to proceed for 20 hours, the samples were titrated in the usual manner. The results are shown in figure 8. The curve showing the action of the protease on raw casein is typical of such action. No peak of activity, besides the optimum around *p*H 6.0, is definitely recognizable, although one might mention that the irregularities occurring around *p*H 4.0 and *p*H 5.0 are not seen on the other curve. On the other hand, the curve representing the activity of the protease on the tryptic digest shows some peculiarities. Besides being much smoother than the first curve, it shows that the optimum reaction was shifted slightly toward the alkaline side, the optimum range lying between *p*H 6.0 and *p*H 7.3, thus bringing the optimum reaction closer to the optimum of trypsin. However, the important fact is that if the protease were of the erepsin type, it should have shown a much higher activity on the tryptic digest than the raw casein sub-

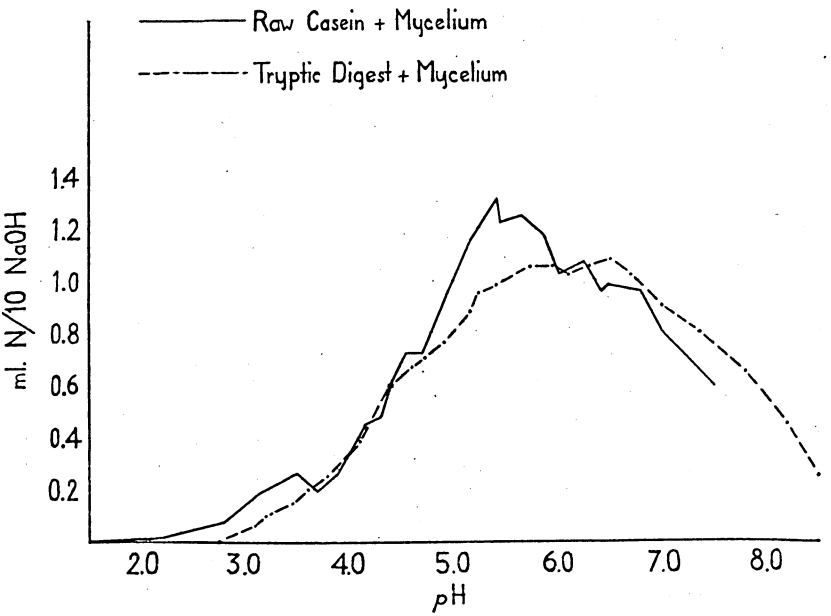

FIG. 8. OPTIMUM ACTIVITY OF PROTEASE ON RAW CASEIN AND ON TRYPSIN-DIGESTED CASEIN

strate, since a tryptic digest is the natural substrate for erepsin. Figure 8 shows that the activity was lower on the tryptic digest. It appeared as if a tryptic enzyme (protease of mycelium) had been allowed to act on a tryptic digest, and that its activity had been inhibited by the products of the first tryptic digestion. This fact points strongly toward the tryptic nature of the protease of *P. roqueforti.* More evidence supporting this idea was provided by the following experiment. Henriques and Sörensen (21) pointed out that the formol titration not only determines the amino groups of the free amino acids but also the titratable amino groups of the polypeptides and even of more complicated protein derivatives. Henriques and Sörensen (21) have tried to obtain, by a new application of the formol titration, more specific information on the nature of the products of proteolysis. For this purpose they devised the formol titration in stages which may be outlined as follows:

first stage: titration to *p*H 8.3.
second stage: titration to *p*H 9.1
Addition of formalin which develops a new acidity
third stage: titration to *p*H 8.3
fourth stage: titration to *p*H 9.1

As shown by the same authors, polypeptides act as stronger acids than amino acids. Therefore, in the first stage of the formol titration the polypeptides are titrated, and the amino acids are titrated afterwards. Henriques and Sörensen used the ratio $\frac{\text{first stage}}{\text{fourth stage}}$ as an indication of the proportions in which the polypeptides and the amino acids existed in proteolytic digests. Pepsin liberates mainly polypeptides. Accordingly, when a peptic digest was titrated, the proportion of polypeptides was larger than in a tryptic digest. As a result, the nature of an unknown enzyme could be determined by the ratio $\frac{\text{first stage}}{\text{fourth stage}}$ when its digest was titrated by the method in stages. Martens (30), using his modification of Foreman's (15) alcohol titration, confirmed the accuracy of the titration in stages as a means of determining the nature of an unknown enzyme. Accordingly, it was decided to try this method. A 2 per cent casein solution was prepared and divided into three lots of 60 ml. each. The reaction in each lot was *p*H 1.5, *p*H 7.8, and *p*H 6.0, respectively. To the first two lots, 0.08 gram of pepsin and trypsin was added, respectively. To the third lot, 0.3 gram of mycelium was added. 10 ml. samples were then withdrawn from each lot and boiled immediately to serve as blanks. Digestion was allowed to proceed for 20 hours after which all the samples were boiled and titrated in stages. It must be emphasized here that the amount of alkali used at each stage is not recorded separately but is added up as the titration progresses. Accordingly, the results obtained at the fourth stage constitute the sum of the milliliters of alkali used for all four stages. Therefore, the ratio $\frac{\text{first stage}}{\text{fourth stage}}$ corresponds to $\frac{\text{polypeptidic N}}{\text{total amino N}}$. Table 6 gives the average titration values of

Table 6. The Formol Titration in Stages and the Nature of Protease

Samples	Stages: first	Stages: fourth	Ratio $\frac{\text{first stage}}{\text{fourth stage}}$
	ml. N/10 NaOH		
Pepsin	.23	.30	$\frac{.23}{.30} = \frac{1}{1.3}$
Trypsin	.73	3.46	$\frac{.73}{3.46} = \frac{1}{4.76}$
Mycelium	.39	1.82	$\frac{.39}{1.82} = \frac{1}{4.66}$

duplicate samples of the digests. As usual, the activity was expressed as the difference between the formol titration of the digests and the formol titration of the blanks, the latter being also titrated in stages.

As seen in table 6, the ratios $\frac{\text{first stage}}{\text{fourth stage}}$ are very characteristic for pepsin and trypsin. They are in close agreement with those given by the authors of the method. This ratio for mycelium is so strikingly close to that of trypsin that it may be regarded as another indication of the tryptic nature of the protease. Henriques and Sörensen (21) and Martens (30) did not give the ratio which would exist in an ereptic digest. However, it is to be expected that if the protease of the mycelium were an erepsin, the ratio $\frac{\text{first stage}}{\text{fourth stage}}$ would have been still larger than it was for the tryptic digest, since erepsin hydrolyzes the peptides to amino acids. Furthermore, the fact that the protease of the mycelium attacks casein, a native protein, may be another argument against the ereptic nature of the protease. The coexistence of a pepsin or trypsin together with an erepsin may be evoked. However, the presence of only one optimum on the reaction-activity curve does not point in this direction.

The wide range of reaction over which the protease of *P. roqueforti* is active supports the idea already suggested (31, 49, 50, 52) that the protease of molds is different from the animal proteases.

The reaction-activity curve of the protease of *P. roqueforti* on a raw casein substrate as well as on a tryptic digest shows only one optimum peak, thus lending support to the idea that only one protease exists in the mycelium. The protease is more active on a peptic digest of casein than on a raw casein substrate. This is thought to eliminate the possibility of the peptic nature of the enzyme. The protease attacks casein, a native protein, which is incompatible with its being of the erepsin type. The protease is less active on a tryptic digest of casein than on a raw casein substrate. This is thought to be an indication that the protease of *P. roqueforti*, being of the trypsin type, was inhibited in its activity by the products of the first tryptic digestion. When a casein solution, which has been digested by mycelium, is titrated in stages, the ratio $\frac{\text{polypeptidic nitrogen}}{\text{total amino nitrogen}}$ confirms the idea that the protease of *P. roqueforti* is of the trypsin type.

STUDIES OF LIPOLYSIS

Optimum Reaction for Lipolytic Activity

The method used in studies of lipolysis has already been described. It should be emphasized that preliminary work showed definitely that reliable results could not be obtained when titration was carried out in an aqueous medium. It was also determined that maximum titration values were obtained when 50 ml. of a 1:1 alcohol-ether mixture were added to 10 ml. of substrate. It should also be mentioned again that titration was carried out directly into the flasks where digestion had taken place, and that lipolytic activity was expressed by the difference between the average titration values of the two digests and of the two blanks.

As mentioned earlier, in the first studies on the reaction favoring the optimum activity of lipase, a phosphate buffer was employed. Some of the curves obtained when this buffer was used are reported in figure 9. Curves 1 and 2 were obtained on a butter oil substrate (3 per cent emulsion), while Curve 3 was obtained on a cottonseed oil substrate (3 per cent emulsion). In the experiments represented by Curves 1 and 2, digestion had proceeded for 16 hours, and for 18 hours in the experiment represented by Curve 3. The striking feature in figure 9 is the width of the optimum range, which extends from *p*H 4.5 up to *p*H 8.0. The same figure also shows that cottonseed oil was hydrolyzed to about the same extent as butter oil. Another feature of figure 9 is the irregularity of the curves. It was thought desirable to use another buffer in order to confirm the optimum reaction obtained and to

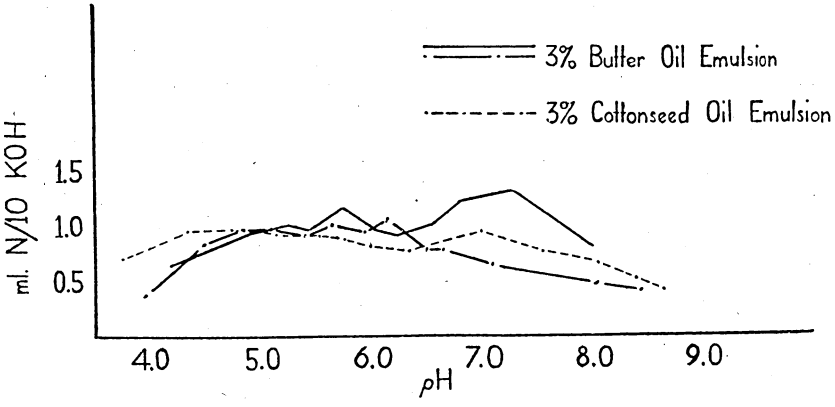

FIG. 9. REACTION FAVORING OPTIMUM ACTIVITY OF LIPASE IN THE PRESENCE OF A PHOSPHATE BUFFER

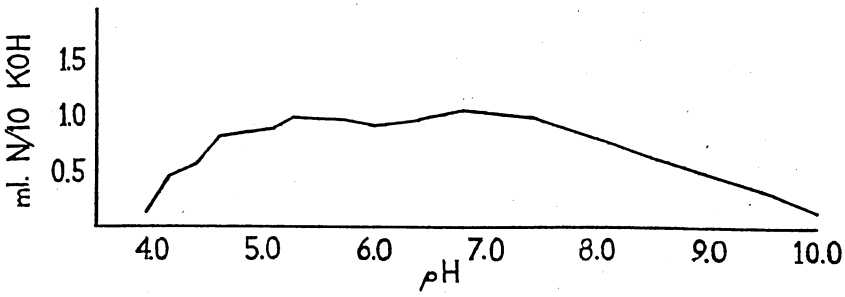

FIG. 10. REACTION FAVORING OPTIMUM ACTIVITY OF LIPASE IN THE PRESENCE OF AN ACETATE BUFFER

attempt to correct the irregularities in the curves. Willstätter and Waldschmidt-Leitz's (54) acetate buffer was selected. The curve obtained with this buffer, after a 15 hour digestion period, is shown in figure 10. It should be noted that it is much more regular than the curves in figure 9. The optimum reaction which was obtained when a phosphate buffer was used is roughly confirmed here, although the optimum reaction seems more definitely confined between *p*H 5.3 and *p*H 7.5.

The literature on the optimum activity of the lipase of molds in relation to reaction is rather meager. Apparently, Mihaéloff (33) found it to be at or close to neutrality. Schenker (39) stated that weak alkalinity retarded, while weak acidity favored, the activity of lipase. The buffer used, if any, is not mentioned by the preceding authors. Kirsh (26), using a phosphate buffer, obtained the optimum activity at *p*H 5.0. It follows that if the findings of these investigators are taken separately, they seem to be in contradiction. However, if they are compared with the results reported in figures 9 and 10, they all fall within the optimum range found by the writers.

The optimum activity of the lipase of *P. roqueforti* was favored over a wide range of reaction, namely, between *p*H 5.3 and *p*H 7.5. Results were more regular when an acetate buffer was used instead of a phosphate buffer.

The Nature of the Esterase

As mentioned earlier, it seems quite well understood that the esterase of molds is not a true esterase but a lipase, although the point is not specifically discussed in the literature. It was thought desirable to make a comparison between the activity of the esterase on esters and on glycerides. The following experiment

Table 7. Comparative Activity of Mold Esterase on Tributyrin and on Ethyl Acetate

Substrates	Total titration		Activity
	Digests	Blanks	ml. N/10 KOH
	ml. N/10 KOH		
Ethyl acetate	1.00	.25	.75
	.99	.25	
	1.01	.25	
Tributyrin	2.61	.32	2.28
	2.61	.33	
	2.61	.34	

was carried out. First, 0.1 M solutions of tributyrin and of ethyl acetate were prepared, and triplicate samples of 10 ml. each were allowed to digest for 12 hours with 0.05 g. of mycelium. The substrates were buffered at *p*H 6.5 with the acetate buffer. Since the tributyrin was not completely soluble in water, 0.1 per cent gum arabic was added to both substrates. Titration was performed as usual. The results, given in table 7, hardly need any comments.

It may be concluded from these data that the esterase of *P. roqueforti* is not a true esterase, but a lipase.

STUDIES PERTAINING BOTH TO PROTEASE AND LIPASE

Influence of the Composition of the Culture Medium on Enzyme Production

There is much contradiction in the literature on the influence of the composition of the culture medium on the production of enzymes by microorganisms. The following experiments were undertaken with the idea of contributing some new data on the subject.

In the first experiment, *P. roqueforti* was grown on the following media:

1. "Standard" medium
2. Czapek's solution
3. Czapek's solution plus 0.1 per cent agar
4. Czapek's solution without sugar
5. Medium 4 plus 3 g. of peptone per liter
6. Medium 2 plus 3 g. of peptone per liter
7. Medium 2 plus 3 g. of peptone plus 3 g. of butterfat per liter

The media were sterilized in the autoclave for 20 minutes at 15 pounds pressure. After cooling they were inoculated with *P. roqueforti,* strain "D," and growth was allowed to develop until the mycelium was covered with spores. As would be expected, the rapidity of growth varied with the different media. Medium

7 produced the most rapid growth (in four days), but the highest yield of mycelium was still obtained on the standard medium. The fat in Medium 7 had not been homogenized into the medium so that it was floating at the surface of the liquid. The mold grew through it, so that when the mycelium was harvested it was greasy and had to be washed with lukewarm water (about 100° F.) in order to remove as much of the fat as possible. However, all of the fat was not thus removed and during the process of drying, the lipase attacked the fat and an intense odor of butyric acid developed. Therefore, part of the lipase was probably inactivated, which was thought to account for the surprising fact that the presence of fat in the culture medium was found to decrease the amount of lipase produced, as shown in table 8 on the following page.

In the second and third experiments the same culture media used in the first experiment were again used and the following added:

8. Medium 4 plus 3 g. of peptone plus 3 g. of butterfat per liter.

The cultural technique was the same as that used in the first experiment. However, the fat, when added, was emulsified into the culture medium by means of a hand homogenizer, using also 0.1 per cent gum arabic to help stabilize the emulsion. At the end of the growth period the mycelium was allowed to dry as usual. No butyric acid odor developed when the mycelium was treated in this way. In order to provide uniform conditions, all the mycelia were extracted the same way. The determinations of proteolytic and lipolytic activity of these mycelia were carried out on the casein substrate adjusted to *p*H 6.0, and the fat emulsion adjusted to *p*H 6.5. Later studies on proteolytic and lipolytic activity were made under the same conditions. The results for the three experiments are reported in table 8. The most striking feature is without doubt the regularly low activity, either proteolytic or lipolytic, of mycelium grown on plain Czapek's solution (Medium 2). Agar when added to Czapek's solution (Medium 3) not only improved growth but also increased materially the production of enzymes. Skim milk added to Czapek's solution together with agar gave the standard medium (Medium 1) which besides giving the highest yield of mycelium also favored a high production of enzymes. Medium 4, when peptone was added to it to give Medium 5, produced mycelium having good proteolytic activity but a higher lipolytic activity than usual, which seems to indicate that sugar in Media 1, 2, and 3 did not retard the pro-

duction of protease but did retard the production of lipase. This is confirmed by results on Medium 6 (which is the same as Medium 5, except that sugar was present), where the amount of lipase produced was decreased. When the first experiment was performed, the decrease in lipolytic activity of the mycelium grown on Medium 7 was thought to be due to a partial inactivation of the enzyme as mentioned earlier. However, when the same peculiarity was observed in the second and third experiments, it became rather puzzling. It appeared as if the addition of fat to the medium decreased the production of lipase by the mold. This is contradicted when one considers the results obtained on Medium 8 where a high production of lipase was obtained. Since this medium also contained fat, it is not reasonable to assume that fat retarded lipase production in Medium 7. On the other hand, Medium 8 did not contain sugar, while Medium 7 did. These results, compared with those obtained on Media 5 and 6, seem to prove that sugar, when present in the culture medium, definitely retards the production of lipase by *P. roqueforti*. The decrease in production of protease on Medium 8 is difficult to explain.

Sugar was found to retard the production of lipase by *P. roqueforti*. Peptone stimulated the production of lipase just as much in the absence of fat as it did in the presence of fat. Plain Czapek's solution does not promote satisfactory growth of *P. roqueforti* and the mycelium grown on it is a poor source of enzymes. The addition of agar to the Czapek's solution not only stimulated growth but also increased the production of enzymes materially.

Table 8. Influence of the Composition of the Culture Medium on Enzyme Production

Culture medium	Activity					
	Protease			Lipase		
	Experiment					
	1	2	3	1	2	3
	ml. N/10 NaOH			ml. N/10 KOH		
1. Czapek + agar + milk (standard)	.91	1.24	1.20	.94	1.03	.80
2. Czapek	.12	.15	.17	.15	.21	.25
3. Czapek + agar	.62	.73	.78	.48	.57	.42
4. Czapek without sugar	*	*	*	*	*	*
5. Medium 4 + peptone	1.18	1.25	1.15	2.35	2.00	2.10
6. Medium 2 + peptone	1.47	1.17	1.30	1.13	1.13	1.00
7. Medium 2 + peptone + fat	1.06	.87	.95	.80	.63	.64
8. Medium 4 + peptone + fat	.....	.63	.60	.....	2.10	2.24

* No growth developed on this medium.

Influence of Age of Cultures on the Enzymatic Activity of the Mycelium

Most of the investigators who have worked on the enzymes of molds have harvested their mycelium when it was covered with spores, stating that the enzyme content of the mycelium was highest at that stage. However, no comparative data are given in the literature. The purpose of the following experiment was to secure such data. To each of 10 Fernbach flasks, 300 ml. of the standard medium were added, and the flasks sterilized in the autoclave for 20 minutes at 15 pounds pressure. After cooling, the media were inoculated with *P. roqueforti,* strain "D." Starting at four days, the mycelium from two flasks was harvested at different intervals and treated in the usual manner. After each sample of mycelium had been ground it was placed in a desiccator over calcium chloride and weighed after it had remained there for two days. The samples were kept in the desiccator until the last sample was ready, and all the determinations were made at the same time. Enzymatic activity was determined by the usual methods using a 20-hour digestion period. The results are given in table 9.

Maximum enzymatic activity of the mycelium was obtained on the sixth day, which is in agreement with the statements found in the literature. However, considering both activity and yield of mycelium, the largest quantity of enzyme units per culture would probably have been obtained after growth had proceeded further.

Comparative Enzymatic Activity of *P. roqueforti* and of Common Molds

The following molds were grown on the standard medium: *P. roqueforti*, strains A, B, C, and D; *A. nidulans*; *H. cladosporioides*; and an unidentified species of Alternaria. The mycelium

Table 9. Stage of Growth and Enzymatic Activity of the Mycelium

Age of cultures	Development of spores	Weight of mycelium	Activity	
Days		Grams	Protease ml. N/10 NaOH	Lipase ml. N/10 KOH
4	slight	1.066	.53	.95
5	medium	1.625	.65	1.13
6	full	2.509	.77	1.11
10	full	4.390	.59	.55
12	full	5.639	.57	.50

Table 10. Comparative Enzymatic Activity of *P. roqueforti* and of Common Molds and of *P. roqueforti* Mycelium and Takadiastase

Organism	Activity	
	Protease	Lipase
	ml. N/10 NaOH	ml. N/10 KOH
P. roqueforti A	.72	.76
B	.91	1.73
C	.56	1.75
D	1.05	1.28
Alternaria sp.	.71	.77
A. nidulans	1.67	.67
H. cladosporioides	1.15	1.19
Takadiastase	1.39	.86
Mycelium	.26	.37

was harvested when covered with spores and its enzymatic activity was determined by the usual methods, using a period of digestion of 20 hours. A comparison was also made between the enzymatic activity of 0.05 g. of an old preparation of takadiastase (age unknown) and the same amount of mycelium from *P. roqueforti*, strain "D," using a three-hour digestion period.

There were wide variations between the enzymatic activity of different strains of *P. roqueforti*. Strain "A" was the least active. Similar variations were noted between different genera of common molds.

Influence of Sodium Chloride on Activity of Enzymes

Salt is added to Roquefort cheese in large amounts, namely, around 4 per cent. Such large quantities of salt have long been suspected of having some regulatory influence in Roquefort cheese. In fact, it was found that salt, in the proportions in which it exists in Roquefort or Blue cheese, checks the growth of undesirable fungi without inhibiting the growth of *P. roqueforti* (42, 45, 46, 47). However, the influence of such concentrations of salt on the activity of the enzymes of *P. roqueforti* has so far remained unstudied. Believing that information of that sort would be at least interesting, if not important, it was thought advisable to devote some time to the subject. Substrates containing different concentrations of sodium chloride were made as follows: to 100 ml. volumetric flasks were added the necessary amounts of salt and of dissolved casein (or emulsified butter oil) and the volume of liquid brought up to the mark. Enzyme activity was then determined on 10 ml. amounts as usual. The results in table 11 show that concentrations of salt up to 4 per cent for protease and

Table 11. Influence of Different Concentrations of Sodium Chloride on the Activity of the Enzymes of *P. roqueforti*

NaCl	Activity	
	Protease	Lipase
Per cent	ml. N/10 NaOH	ml. N/10 KOH
0	1.05	1.08
1	1.23	1.20
4	1.19	1.19
6	1.01	1.14
10	.61	.97
15	.39	.81
20	.20	.52

6 per cent for lipase do not retard the action of the enzymes; some activation can even be noted. From there on, enzymatic activity, especially of protease, rapidly decreases as the concentration of the salt increases.

Stage of Growth at Which the Enzymes of *P. roqueforti* Appear in the Culture Medium

Some enzymes have been classified both as endo- and exo-enzymes, depending on the investigator and on the organism investigated. Dox (14) was of the opinion that all enzymes, to varying extents, were released from the mycelium at the time of sporulation. Before sporulation they were supposed to be of the endoenzyme type.

The purpose of the following experiment was to determine whether the enzymes of *P. roqueforti* were released into the culture medium and, if they were, at what stage of the growth. The first method used was a modification of Hédon's (20) quick test, which has been described elsewhere. A number of growing cultures were tested every day for the presence of enzymes in the culture medium. After 12 days, negative results were still obtained. It was thought that the method used was not sensitive enough. Accordingly, a new series of experiments was started. The mold was grown on two media: the standard medium and a medium where the skim milk was replaced by 3 g. of peptonized milk per liter. Spores on the latter medium developed more quickly (two days as compared with four days) and more abundantly than on the standard medium. At different intervals, determinations were made by the usual methods (formol titration and titration of free fatty acids), using 1 ml. of culture medium in 10 ml. of substrate and allowing digestion to proceed for 36 hours. The last determination was made when the cultures

Table 12. Appearance of Enzymes in the Culture Medium

Age of cultures	Enzymatic activity of 1 ml. of culture medium			
Days	Medium			
	1*		2†	
	Protease ml. N/10 NaOH	Lipase ml. N/10 KOH	Protease ml. N/10 NaOH	Lipase ml. N/10 KOH
4	.00	.00	.00	.00
5	.00	.00	.00	.00
10	.00	.02	.00	.01
17	.00	.05	.00	.04
30	.08	.12	.04	.05

* Standard medium.
† Medium containing peptonized milk.

were 30 days old. At that stage the mycelium was beginning to disintegrate. According to the results presented in table 12, protease was beginning to be released into the culture medium only when autolysis was occurring in the mycelium. The lipase was released sooner but not in considerable amounts. These results were rather surprising when compared with the easy extraction of the enzymes of certain molds, such as Aspergillus and other species of Penicillium, as reported in the literature (5, 6, 19, 35, 43). Accordingly, it was thought desirable to confirm those results by another method and make comparisons with other molds before drawing any conclusions. The following method was adopted:

Protease—Petri plates were poured with standard nutrient agar containing 5 per cent of skim milk. After the agar had hardened, the plates were inoculated at the center with the organisms, so as to allow giant colonies to develop. At different intervals the plates were examined and observations made on the appearance of the clear zone around the colonies.

Lipase—The copper soap method of Berry (4) was followed.[3] Enough plates were inoculated with each organism to provide for three determinations.

As shown by table 13, the results obtained by the first two methods were confirmed when the plate method was used. Table 13 also shows that the enzymes of the other molds were readily liberated from the mycelium. Accordingly, these experiments

[3] This method is briefly as follows: 5 ml. of butter oil are added to 100 ml. of nutrient agar. After sterilization the bottles are shaken thoroughly in order to bring about emulsification of the oil, and the agar is poured in petri plates. After the agar has solidified, the plates are inoculated by the streak method and incubated for suitable lengths of time. After incubation the plates are flooded with a saturated aqueous solution of copper sulphate for 10 minutes and rinsed gently with water. When the fat has been attacked, conspicuous bluish-green streaks of insoluble copper soap appear on the plates.

Table 13. Liberation of Enzymes from the Mycelium Checked by the Plate Method

Organism	Extent of clear zone			Copper soap formation		
	Protease			Lipase		
	after					
	3 days	5 days	10 days	3 days	5 days	10 days
P. roqueforti A	x	x	x	—	—	x
B	x	x	x	—	—	x
C	x	x	x	—	—	x
D	x	x	x	—	—	x
A. nidulans	xx	xxx	xxxx	—	x	xx
A. niger	xxx	xxxx	xxxx	x	xxx	xxxx
H. cladosporioides	xxx	xxxx	xxxx	—	x	xx
Alternaria sp.	xxx	xxxx	xxxx	—	x	xx

Code:	Clear zone (width around colony)	Copper soap formation (extent of blue color)
—	none	none
x	just visible	slight
xx	about 1 cm.	medium
xxx	1-3 cm.	marked
xxxx	more than 3 cm.	very marked

indicate that the enzymes of *P. roqueforti* are not readily liberated from the mycelium. Should it be concluded that they are of the intracellular type? Sufficient evidence is not available to permit definite conclusions. More information on this subject is to be found in the data in table 13.

Isolation of the Enzymes of *P. roqueforti*

Among the goals set at the start of the present work was that of the preparation of the enzymes of *P. roqueforti* in a concentrated form by precipitating them from their solutions. However, difficulties of an unexpected complexity were encountered when it came to the extraction of the enzymes. These difficulties, together with a lack of time, are responsible for failure to complete this part of the work.

All of the work done on this subject, save one attempt to precipitate the enzymes, has been on the extraction of the enzymes from the mycelium. It is not the purpose to report in detail the numerous experiments which have been carried out in this connection and which all point to the same conclusions. It is felt, rather, that an outline of the work done should be given, with a more detailed report of the last experiment.

In the first experiment made with the ultimate purpose of precipitating the enzymes, mycelium powder was allowed to soak for five hours in distilled water, mycelium and water being in proportions such that 1 ml. of water would correspond to 0.05 g.

of mycelium (these proportions were observed in all the experiments pertaining to this subject). After filtration a determination was made of the comparative activity of 1 ml. of filtrate and 0.05 g. of mycelium. The experiment, repeated three or four times, showed that the filtrate had only very weak, or no, proteolytic or lipolytic activity. It was then suspected that the enzymes might have been adsorbed by the filter paper. To verify this a number of samples of mycelium were allowed to macerate in distilled water, the reaction in the different mixtures being brought to different levels. These mixtures, after soaking, were divided into four portions of which three were filtered through filter paper of different make. In the fourth portion the mycelium was separated by centrifugation. When activity determinations were made on the different extracts, all were found to be still inactive. Since the extracts which had been centrifuged were also inactive, this was considered to have proved that the inactivity of the other extracts was not due to the fact that the enzymes had been adsorbed by the filter paper. This experiment also showed that the reaction at which maceration was taking place had no influence on the liberation of the enzymes by the mycelium. Furthermore, the extracted mycelium was found to be still as active as unextracted mycelium, proving that the enzymes were not inactivated during the extraction process, but were simply not liberated from the mycelium. The filtrate from mycelium treated by the "acetondauerhefe" method and the press juice from mycelium powder which had been allowed to soak for some time were still inactive or nearly so. Different salts, as suggested in the literature, when added to the water gave no better results than when pure water was used as extractant. Finally, the best results were obtained when the mycelium powder was thoroughly ground with sand in a mortar. In the last experiment of this series, 20 g. of sand-treated mycelium were allowed to soak in 400 ml. of distilled water for four hours. A 10 ml. portion of the filtrate was kept for comparative studies, and the remainder was half-saturated with ammonium sulphate, which gave rise to a light, white, flocculent precipitate. The mixture was then centrifuged for 30 minutes. However, the extract, even after filtration, was not perfectly clear, so that during centrifuging, the impurities rose to the surface of the liquid in the tubes. These impurities, which will be called "skimmings," were removed by suction and kept separately. The largest part of the liquid in the tubes was then eliminated by decantation, and the sediment recovered on a filter paper. The precipitate, after drying, had a yellowish color

and the amount was too small to allow for purification procedures. To test its enzymatic activity, the filter paper was divided into four equal parts which were introduced directly into the substrates, thus allowing for one protease and one lipase digest and one blank for each. The enzymatic activity (after a 16-hour digestion period) of the different portions obtained throughout this experiment are given in table 14. They show that the enzymatic activity of the 1 ml. extract (corresponding to 0.05 g. of mycelium) was only one third, or less, of the enzymatic activity of the original mycelium. The extracted mycelium was still quite active, proving that the enzymes were not destroyed by grinding, but that only a small portion was liberated. (In other experiments, where maceration was allowed to proceed for longer periods of time, the same results were obtained.) Surprisingly enough, the precipitate collected on the filter had no proteolytic activity. (Lipolytic activity could not be determined, because the ammonium sulphate retained by the filter made it impossible to obtain a definite end point with phenolphthalein.) On the other hand, the proteolytic activity (lipolytic activity could not be determined for a reason similar to the above) of the skimmings was quite high. It must be emphasized, however, that the figures expressing the activity are not very accurate, because the skimmings contained large amounts of ammonium sulphate, which ammonium sulphate was also titrated by the formol titration method. Very high figures were obtained from the titration of both digests and blanks, which means that small errors in measuring the volumes of skimmings might have caused differences greater than the figures representing the activity. However, that digestion had been extensive could be ascertained by the fact that when the formalin was added, it caused precipitation of casein (the reaction being brought as low as *p*H 4.0, due to the acidity developed by the reaction between the formaldehyde and the

Table 14. Enzymatic Activity of the Different Portions of Material Obtained in an Enzyme Precipitation Process

Portion	Activity	
	Protease	Lipase
	ml. N/10 NaOH	ml. N/10 KOH
Extract (1 ml.)	.16	.08
Skimmings (1 ml.)	2.42	.....
Precipitate (on filter paper)	.00	.....
Extracted mycelium*	.25	.50
Original mycelium	.48	.54

* This is the wet sand-treated mycelium after extraction. The sample was not weighed, but amounts thought to correspond approximately to 0.05 g. of dry mycelium were used.

ammonium salt) in all the samples containing ammonium sulphate (i.e., the blanks and the samples where the filter paper had been introduced) except in the samples digested by the skimmings. It seems, therefore, that during centrifuging, the enzymes had been carried to the surface by the impurities present in the extract. Because of lack of time, purification of the skimmings could not be carried any further.

Further studies on the subject should be made in order to find a means of extracting the enzymes, which have proved themselves very difficult to obtain in solution. The reason for this stubbornness cannot be given yet. After treating the mycelium according to the procedures followed for extraction of both exo- and endoenzymes, the mycelium still held its enzymes. Accordingly, the theory of exo- and endoenzymes proves insufficient to explain this phenomenon. It seems as if the enzymes were tenaciously held on the walls of the cells, either inside or outside, and that they may be liberated only when the mycelium tissue is submitted to a drastic treatment—such as grinding with sand—which destroys, to a certain extent, the adsorbing tissues. The use of surface tension-decreasing agents might prove beneficial.

The enzymes of *P. roqueforti* have proved very difficult to obtain in solution. Their status cannot be satisfactorily explained by the usual theory of exo- and endoenzymes.

Protease was precipitated when its solution was half saturated with ammonium sulphate.

PRACTICAL APPLICATION TO CHEESE RIPENING

Some investigators have attempted to shorten the ripening period for Blue cheese by allowing the cheese to ripen at higher temperatures (32), by homogenizing the milk (23, 27, 28, 29), or by adding lipase to the milk or to the curd (8, 23). Homogenization of the milk so far has proved to be a quite helpful means of hastening the ripening of the cheese. Curing at higher temperatures produced cheese of inferior quality, and the addition of lipase to the milk or to the curd caused the development of a bitter flavor. It is now understood that the development of the typical flavor in Blue cheese is not solely a matter of hydrolysis of the fat. Fat hydrolysis is only the first step in that process. The most important step is a biochemical process, whereby the mold, due to special physiological aptitudes, oxidizes the liberated fatty acids to methylketones which seem to be the aromatic compounds (18, 41). It follows, therefore, that attempts at hastening

the development of aroma should be concentrated on hastening the development of the aroma-producing agent, namely, the mold. Furthermore, ripening of Blue cheese is not concerned solely with the development of the typical flavor and aroma, but also with the breaking down of the curd. Accordingly, those two factors, flavor development and breaking down of the curd, must be coordinated in all attempts at hastening the ripening of Blue cheese. The addition of enzymes to the curd should hasten the development of *P. roqueforti* by making quickly available to the mold more simple products, more readily adsorbable than the complex molecules of paracasein. Since the ripening of cheese is concerned with protein hydrolysis as well as with fat hydrolysis, it seems reasonable to conclude that both protease and lipase should be added. It was pointed out in another section that the enzymes of *P. roqueforti* and of microorganisms in general seem to be different from animal enzymes. Perhaps they also work differently. The products due to the activity of animal enzymes, especially proteases, might be undesirable in cheese. Likewise, the enzymes added must be active at the reaction existing in cheese.

It was thought that studies on the hastening of the ripening of Blue cheese should be undertaken as a normal sequence to the enzyme studies already reported. In agreement with the above considerations, it was also thought that the enzymes to be added should be those present in normal cheese. Accordingly, it was planned to incorporate in the curd, the enzymes of *P. roqueforti,* using mycelium as the source of enzymes. It was also planned to incorporate the enzymes in the curd in a more concentrated form, but failure to obtain the enzymes in the desired form caused this phase of the work to be abandoned.

Three batches of experimental Blue cheese were made as follows: the usual procedure used by the Dairy Division of the University of Minnesota was followed up to the time of hooping. At that stage, the amount of curd necessary for the experimental cheeses was withdrawn, and the mycelium mixed with it. The mycelium-curd mixture was then hooped, mold powder being added as in normal cheeses. The remainder of the curd in the vat was hooped as usual. The whole batch of cheese was then treated as an ordinary batch. From time to time observations were made on the cheeses and information on the degree of ripening was obtained by making determinations of amino nitrogen, fat hydrolysis, and pH. As controls, three of the normal cheeses in each batch were used. Representative samples were taken from each cheese and the samples from each group of cheeses

were mixed intimately together by grinding in a mortar, so that the analyses were made on a sample representing all the cheeses of the given group. Duplicate determinations were made, and the average of the results was taken. Table 15 gives an outline of the results obtained. In this table the intensity of the typical aroma developed is indicated by the following code:

Typical Aroma

— not detectable	xxx pronounced
x detectable	xxxx abundant
xx appreciable	

However, the differences between the experimental cheeses and the controls were much more marked than the figures in the columns for amino nitrogen and fat hydrolysis would indicate. The experimental cheeses in the first batch, after 160 days, were very fine. The typical flavor was well developed, and those who tasted them agreed that they had attained the same degree of maturity as normal cheeses in 10 months. The controls, on the other hand, still tasted green and were far from being ready for the trade. The mold in the experimental cheeses grew throughout the mass of the cheese, while in the controls, growth took place mainly at the center. It might be asserted that the greater development of *P. roqueforti* in the cheeses was due to the very heavy inoculation constituted by the mycelium added. However, researches by other investigators (29) have shown that there was no advantage in using more than 0.01 per cent of mold powder for inoculation of the cheeses. It should also be emphasized that from the point of view of protein breakdown, there was no significant difference between the experimental cheeses and the controls. Evidently, the mold is mainly responsible for the development of aroma. There is no proof, however, that if the protease had been added to the cheese in a more readily available form, ripening would not have proceeded still faster.

Addition to Blue cheese of the enzymes of *P. roqueforti* in the form of mycelium produced cheeses of fine quality and ready for the trade in about five months, as compared with 10 months for normal cheeses.

There seemed to be no great advantage in using more than 6 g. of mycelium per five-pound cheese.

The addition of the enzymes of *P. roqueforti* to Blue cheese has proved beneficial. However, since the enzymes were added in the form of mycelium, and since studies reported here have shown that the enzymes are not readily liberated from the mycelium, one is allowed to assume that if the enzymes had been

Table 15. Results Obtained on Three Batches of Blue Cheese, When Mycelium Was Added to the Curd

Batch No.	Mycelium added to cheese	Age	Amino nitrogen	Fat hydrolysis	pH	State of curd	Typical aroma	Mold growth
	grams	days	mgm. NH_2-N p.gm.ch.	ml. N/10 NaOH*				
1	6	26	3.91	.....	4.72	Breaking	x	Clearly visible
		62	6.23	10.2	5.48	Quite broken	xxx	Abundant
		160	11.67	23.8	6.1	Broken	xxxx	Very abundant
	None	26	3.25	.....	4.60	Green	—	Not visible
		62	5.55	7.6	5.38	Quite broken	x	Slight
		160	11.04	16.9	5.55	Broken	xx	Medium
2	6	26	3.79	.....	4.48	Breaking	—	Visible
		62	3.92	6.7	5.00	Breaking	xx	Medium
		140	9.66	19.8	5.92	Broken	xxxx	Very abundant
	12	26	4.40	.....	4.50	Breaking	x	Clearly visible
		62	4.56	7.5	5.15	Breaking	xxx	Abundant
		140	8.06	20.5	5.82	Broken	xxxx	Very abundant
	None	26	3.56	.....	4.60	Green	—	Not visible
		62	3.72	6.0	5.20	Breaking	x	Slight
		140	7.24	15.7	6.20	Broken	xx	Medium
3	6	26	4.50	.....	4.55	Breaking	x	Visible
		62	4.66	6.1	5.55	Breaking	xx	Abundant
		123	7.44	15.0	5.78	Quite broken	xxx	Very abundant
	8.5	26	4.11	.....	4.60	Breaking	x	Visible
		62	4.80	5.3	5.47	Breaking	xx	Abundant
		123	7.80	16.7	5.80	Quite broken	xxx	Very abundant
	None	26	4.11	.....	4.55	Breaking	x	Visible
		62	4.40	3.2	5.23	Breaking	x	Slight
		123	6.43	9.8	5.95	Quite broken	xx	Medium

* ml. of N/10 NaOH required to neutralize the steam distillate from 50 g. of cheese.

added to the curd in a more readily available form, the results would have been still better. Accordingly, it would be desirable that further studies be instituted on the enzymes of *P. roqueforti*. Emphasis should be laid upon the extraction of the enzymes and on their isolation.

Conclusions

1. While some of the common molds may thrive on Czapek's solution, *P. roqueforti* grows very poorly on this medium. Growth is diffuse and the mycelium can be recovered only by means of filtration.

2. Although some nitrogenous compounds stimulate growth markedly, none of those tested favors the formation of a felt at the surface of the medium (which is the type of growth desired for a high mycelium yield and an easy recovery of the mycelium).

3. Sodium nitrate, the source of nitrogen used in standard Czapek's solution, is one of the poorest sources of nitrogen that can be used as far as *P. roqueforti* is concerned.

4. A strong pellicle is consistently formed when agar, in 0.1 per cent concentration, is added to the medium.

5. A medium, which is a modification of Czapek's solution, has been devised on which the mycelium yield by *P. roqueforti* is far superior to that obtained on any other medium tried.

6. The reaction of the medium (within certain limits) and the source of carbon used do not influence significantly the growth of *P. roqueforti* on Czapek's solution.

7. *P. roqueforti* does not grow readily on a medium having a high oxidation-reduction potential. An initial oxidation-reduction potential below 400 millivolts definitely favors the growth of the organism and the formation of a pellicle.

8. Agar in concentration of 0.1 per cent decreases the initial oxidation-reduction potential of Czapek's solution below 400 millivolts.

9. The yield of mycelium, on different media, has been found to be inversely proportional to the initial oxidation-reduction potentials of the media.

10. It is possible that when agar and an organic source of nitrogen are added to Czapek's solution, the improvement in growth of *P. roqueforti* may be due to a combination of factors

such as lower initial oxidation-reduction potentials, increase in viscosity of the medium or provision of surface where growth is catalyzed, and an immediate, more available source of nitrogen.

11. As a measure of proteolysis, the formol titration, when the end points of titration are determined by means of the glass electrode, gives results that are consistent and in close agreement with those obtained by the Van Slyke manometric method.

12. The protease of *P. roqueforti,* on a 1 per cent casein substrate and when phosphate buffer is used, reaches its optimum activity in a range lying between *p*H 5.8 and *p*H 6.3.

13. Toluene has been found to be a poor antiseptic when a long digestion period is used in studies of proteolysis by *P. roqueforti.* A period of digestion longer than 20 hours is not recommended.

14. *P. roqueforti* seems to produce only one protease. This enzyme appears to be of the trypsin type, however, being different from animal trypsin in that its optimum activity occurs at a lower *p*H and over a wider range of reaction.

15. The optimum activity of the lipase of *P. roqueforti* is favored over a wide range, namely, between *p*H 5.3 and *p*H 7.5, when the substrate is a 3 per cent butter oil emulsion and in the presence of an acetate buffer.

16. The esterase of *P. roqueforti* is not a true esterase but a lipase.

17. Sugar, when present in the culture medium, has been found to retard the production of lipase. Agar and organic nitrogenous compounds increase the production of both protease and lipase, as compared with that obtained on plain Czapek's solution.

18. Maximum enzymatic activity of the mycelium is obtained as soon as the cultures have attained the stage of full sporulation.

19. Enzyme production by *P. roqueforti* may vary widely from strain to strain.

20. Sodium chloride, in concentrations such as those existing in Blue cheese, does not retard the action of the protease and lipase of *P. roqueforti.*

21. The enzymes of *P. roqueforti* have proved very difficult to obtain in solution. Their nature cannot be satisfactorily explained by the usual theory of exo- and endoenzymes. It seems as though they were tenaciously adsorbed on the walls of the cells, and that they may be liberated only when the mycelium tissue is submitted to a drastic treatment.

22. The protease of *P. roqueforti* is precipitated when its aqueous solution is half saturated with ammonium sulphate.

23. The addition of the enzymes of *P. roqueforti,* in the form of mycelium, to Blue cheese (in the proportion of six g. of mycelium per five-pound cheese) at the time of hooping produces cheeses of fine quality and ready for the trade in about five months, as compared with 10 months for normal cheese.

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